

# **Report of the 2009 Proficiency Test of the Community Reference Laboratory for Mycotoxins, for the Network of National Reference Laboratories, Regarding the Determination of T-2 and HT-2 Toxins in Cereal Products**

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## Summary

A proficiency test was conducted by the Community Reference Laboratory for Mycotoxins with 29 European National Reference Laboratories (NRLs) for Mycotoxins and one laboratory from a candidate country. The materials shipped were a solution of known T-2 and HT-toxins content in acetonitrile and three cereal test materials with unknown levels of T-2 and HT-2 toxins. Laboratories determined the content of T-2 and HT-toxins mainly by either enzyme linked immuno-sorbent assay (ELISA), gas chromatography (GC-MS) or high-performance liquid-chromatography (HPLC) followed by fluorescence or mass selective detection (MS). From each Member State (MS) one NRL reported results, with two MS reporting results from two different NRLs, one for feed and one for food.

Applying the Horwitz equation as a basis for setting the target standard deviation for proficiency (19.6% for T-2 toxin and 21.5% for HT-2 toxin in the spiked test material), resulted in 21 out of the 30 laboratories reporting satisfactory z-scores for T-2 toxins and 15 laboratories reporting satisfactory ones for HT-2 toxins after recovery correction. Two laboratories did not send in results for HT-2 toxin, but only for T-2 toxin. Four laboratories reported questionable results within a z-score limit of 2 to 3 for T-2 toxin and 2 laboratories for HT-2 toxin. The remaining laboratories reported z-scores above 3, which are unsatisfactory. Taking the  $\zeta$ -score as benchmark for the sum of T-2 and HT-2 toxin, the number of satisfactory results reduced to 14. No z-scores were calculated for the low contaminated and the high contaminated material.

## Introduction

In 2006 the Institute for Reference Materials and Measurements (IRMM) in Geel was designated as Community Reference Laboratory (CRL) for Mycotoxins by the Directorate General for Health and Consumers (DG SANCO). One of the main responsibilities of the CRL is to organise comparative testing to benchmark and harmonise the measurement capabilities of National Reference Laboratories (NRLs) working in the same field. The topic of the PT2009 was the determination of T-2 and HT-2 toxins in cereals.

Test materials in this study were wheat based cereal flours, either free of, naturally contaminated or fortified with T-2 and HT-2 toxins. Contaminated batches were tested for homogeneity using an ANOVA based experimental design and found to be sufficiently homogeneous. The stability of the test material was not tested explicitly, as the material was intended to be used shortly after preparation in the proficiency test.

## Methodology

Each participant received the following test materials:

- 3 coded test materials with a level of T-2 and HT-2 toxins unknown to the participants of which one contained T-2 and HT-2 toxins at a level less than 20 ng/g, one that was fortified to represent a material as it can be expected to be relevant for decision making in the region of a prospective legislative limit. The third sample was highly contaminated above 6 mg/kg for the sum of T-2 and HT-2 toxins.
- 1 ampoule of a test solution of "T-2 and HT-2 toxins in acetonitrile" with an indicated concentration of T-2 and HT-2 toxins.

Participants were asked to measure the 3 coded cereal test materials and report the results. Further they were asked to report their recovery rate and how they reported the values (corrected or uncorrected for recovery).

The instructions as sent to the participants are included in the annex.

Graphs were made with MS-Excel® or SigmaPlot 9.01. Results were gathered via electronic forms using Adobe Life-cycler. z-scores and  $\zeta$ -scores were calculated using Microsoft Excel®.

The target standard deviation ( $\sigma_p$ ) for the calculation of z-scores for T-2, HT-2 and the sum of T-2 and HT-2 toxins were calculated from the Horwitz function. These parameters were derived from the following formulas:

$$(1) \quad (\sigma_p) = 0.02^{c \times 0.8495}; \text{ where } c \text{ is the mass fraction of the analyte in the sample}$$

$$(2) \quad z - score = \frac{x - \mu}{\sigma_p}; \text{ where } x \text{ is the reported value and } \mu \text{ the reference value}$$

$$(3) \quad \zeta - score = \frac{x - \mu}{\sqrt{(u_x)^2 + (u_\mu)^2}}; \text{ where } u_x \text{ and } u_\mu \text{ are the uncertainties (k=1) associated with } x \text{ and } \mu.$$

As a result,  $\sigma_p$  was 50.8  $\mu\text{g/kg}$  (19.6% RSD) for T-2 and 29.8  $\mu\text{g/kg}$  (21.5% RSD) for HT-2 for the spiked material. A z-score for the sum of both toxins was calculated. Despite the shortcuts of this

approach, we considered this a fit for the purpose approach to obtain a useful benchmark parameter. For the sum of both toxins, ( $\sigma_P$ ) was calculated as 73  $\mu\text{g/kg}$  (18.4% RSD).

## Results and Discussion

### *Assignment of values*

The assigned values were determined by an isotope dilution mass spectrometry procedure using  $^{13}\text{C}$  labelled T-2 and HT-2 toxins. The levels for the sum ( $\Sigma$ ) of T-2 & HT-2 were 16.9  $\mu\text{g/kg}$  for the low level, 397.1  $\mu\text{g/kg}$  for the medium level and 6787  $\mu\text{g/kg}$  for the high level. The test materials were naturally contaminated in the case of the low level (LO) and high level (HI) material, the medium level material was spiked blank (SP) material. Details of the certification procedure and on the uncertainties as well as the levels for T-2 and HT-2 toxins are given in the Annex.

### *Benchmarking performance by z-scores*

For the NRL benchmarking, only the z-scores of the SP material were used as these levels are in the range of future legislative limits.

In contrast, the LO material was in the region where the limit of quantification (LOQ) of some methods could be expected and below anticipated future legislative limits, while the HI material was contaminated with a rather high level of HT-2 toxin, which was thought to be likely outside the working range of most methods.

This exercise allows some reflection on how participants deal with a situation when results are at (or beyond) the extremes of the working ranges of their analytical methods. Taking into account the details that participants have on the working range of their methods this information can assist in verifying suitable procedures when results are generated at the extremes of working ranges. Details are not discussed here, but shall be reviewed by participants themselves on the basis of the internal data available.

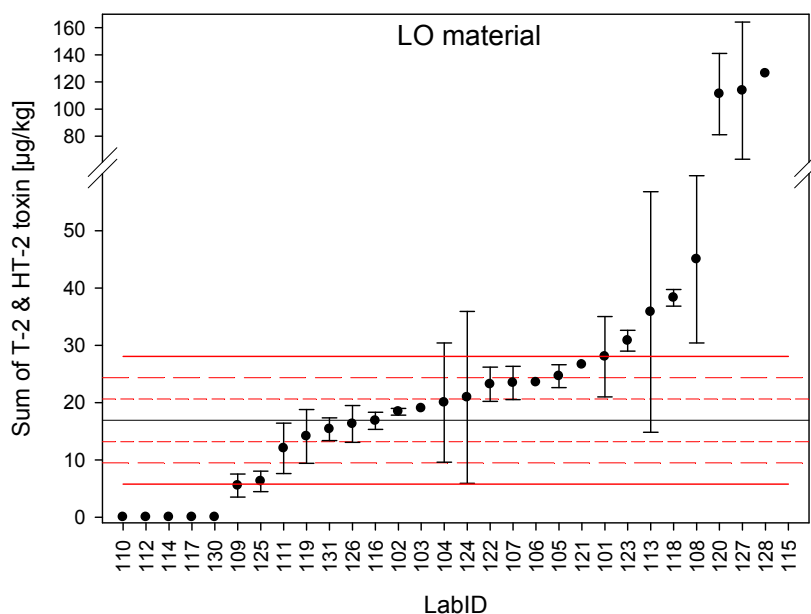
Figures 1 to 3 show the results of each participant for the sum of T-2 and HT-2 toxins which were used for the z-scoring. Additional graphical information on the individual T-2 and HT-2 results is given in the Annex (see Figures I - III). This additional information shows that some participants reported higher results for T-2 and lower results for HT-2 (laboratories 109, 106 and 108) for the LO material (see Annex, Figure I). The cause for this is difficult to identify, but the respective participants are strongly advised to cross check signal identities or correct identity in their reporting, while this has no effect on the z-scoring for the sum of both toxins. The same holds true for laboratories 120, 109 and 127 (see Annex, Figure II), where the opposite is the case (higher HT-2 and lower T-2 levels). The results depicted in Figure III of the Annex suggest that the quantification of the rather high levels of HT-2 caused the underestimation of this analyte, while the lower levels for T-2 did not. At even lower levels (see Annex, Figure II) such an underestimation was not observed at all.

The resulting z-scores for the SP material are tabulated in Table 1 taking the reference value from the IDMS process as assigned value. The standard deviations for the proficiency assessment ( $\sigma_P$ ) were derived from the Horwitz function. They were 50.8  $\mu\text{g/kg}$  (19.6% RSD) for T-2 toxin and 29.8  $\mu\text{g/kg}$  (21.5% RSD) for HT-2 toxin, respectively. Four participants obtained doubtful results for T-2 toxin and another five obtained unsatisfactory results. For HT-2 toxin two results were doubtful and eleven results were unsatisfactory, while two participants missed to report results for HT-2 toxin. With one exception, all participants that reported doubtful or unsatisfactory results for T-2 toxin also reported at

least doubtful results for HT-2 toxin. This indicates that the overall performance of the population for T-2 toxin is better than the performance for HT-2 toxin.

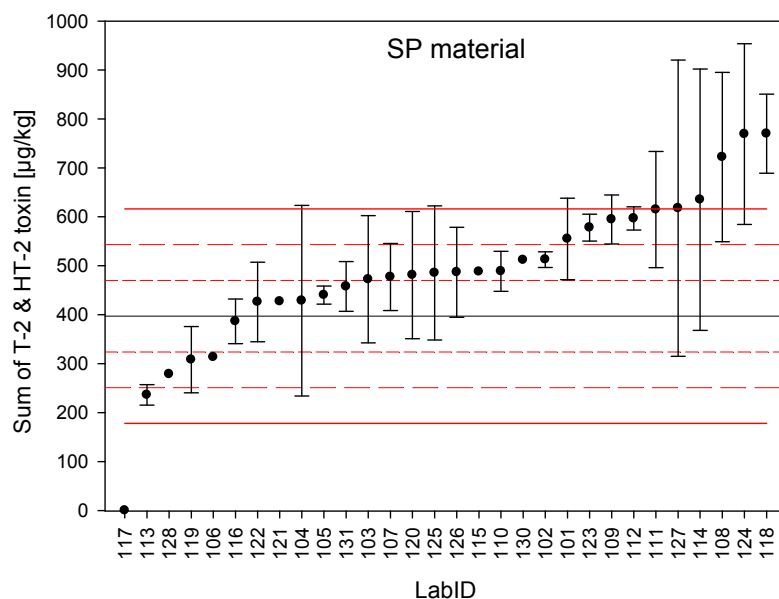
When results were benchmarked using  $\zeta$  scores, only the  $\zeta$ -score for the sum of T-2 and HT-2 toxins was used. The reason for this was that only the uncertainty value for the combined toxins was asked and therefore reported. Consequently the number of doubtful results was calculated as 3 and the number of unsatisfactory results as 13. This means that  $\zeta$ -scoring resulted in at least doubtful results for more than half of the participating laboratories. The reason is partially due to the reported estimates for measurement uncertainty and the fact that the calculated divisor  $\sqrt{(u_x)^2 + (u_\mu)^2}$  resulted in smaller values than the  $(\sigma_p)$  value used for z-scoring. For example the result for the sum of T-2 and HT-2 of participants 111 and 127 did not differ much, while their uncertainty statements did. As a result the uncertainty range did in one case overlap with the reference value from the IDMS process (127) and in another not (111). Therefore the score for participant 127 was found satisfactory and the one for 111 was classified as unsatisfactory. In this case no attention was paid to whether the uncertainty was in agreement with the *fit-for-purpose* function given in Commission Regulation (EC) No 401/2006. This example illustrates that when benchmarking using  $\zeta$ -scores, additional care regarding the way of data reporting must be taken.

Figure 1: Reported results for the LO material



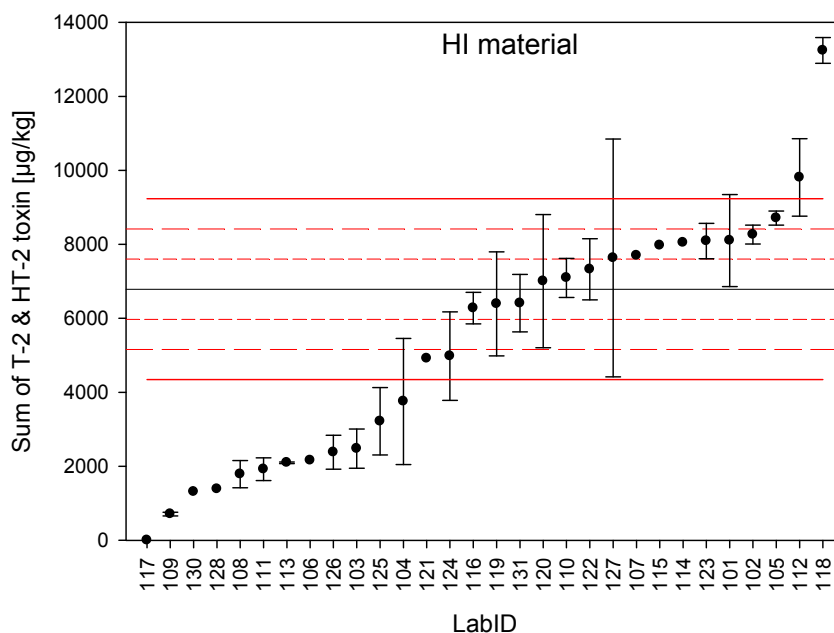
Results for the sum of T-2 and HT-2 as black dots (●). Bars indicate the reported measurement uncertainty. The black line is the reference value from the certification study (16.9 µg/kg). The short dashed red lines reflect the limits of the target standard deviation calculated from the Thompson-Horwitz function, assuming a maximum relative standard deviation of 22% below concentrations of 120 µg/kg. These limits reflect a z-score of |1| (13.2-20.6 µg/kg). The long dashed red lines, reflect the z-score limit of |2| (9.5-24.3 µg/kg). The solid side-shortened red lines reflect the z-score limit of |3| (5.8-28.1 µg/kg).

Figure 2: Reported results for the SP material



Results for the sum of T-2 and HT-2 as black dots (●). Bars indicate the reported measurement uncertainty. The black line is the reference value from the certification study (397 µg/kg). The short dashed red lines reflect the limits of the target standard deviation calculated from the Thompson-Horwitz function (18.4% RSD). These limits reflect a z-score of |1| (324-470 µg/kg). The long dashed red lines, reflect the z-score limit of |2| (251-543 µg/kg). Results outside this range are classified as questionable. The solid side-shortened red lines reflect the z-score limit of |3| (178-616 µg/kg). Results outside this range are classified as unsatisfactory.

Figure 3: Reported results for the HI material



Results for the sum of T-2 and HT-2 as black dots (●). Bars indicate the reported measurement uncertainty. The black line is the reference value from the certification study (6787 µg/kg). The short dashed red lines reflect the limits of the target standard deviation calculated from the Thompson-Horwitz function (12% RSD). These limits reflect a z-score of |1| (5973-7601 µg/kg). The long dashed red lines, reflect the z-score limit of |2| (5158-8416 µg/kg). The solid side-shortened red lines reflect the z-score limit of |3| (4344-9230 µg/kg).



Table 1: Summary of z-scores and  $\zeta$ -scores for the SP material

Lab ID	z-score T-2 toxin	z-score HT-2 toxin	z-score (T-2 & HT-2)	$\zeta$ -score (T-2 & HT-2)
117	-5.1	-4.6	-5.4	-12.1
120	-2.8	7.5	1.1	1.2
119	-1.5	-0.4	-1.2	-1.9
127	-0.7	8.6	3.0	1.4
113	-0.4	-	-	-11.5
116	-0.4	0.2	-0.1	-0.3
109	-0.3	7.1	2.7	4.8
106	0.2	-3.1	-1.1	-2.6
122	0.2	0.6	0.4	0.6
107	0.4	2.0	1.1	1.7
128	0.4	-	-	-12.1
121	0.5	0.2	0.4	0.9
105	0.7	0.3	0.6	1.3
126	0.7	1.8	1.2	1.6
103	0.8	1.2	1.0	1.0
131	1.0	0.4	0.8	1.5
104	1.1	-0.8	0.4	0.3
115	1.1	1.1	1.2	2.8
125	1.2	0.8	1.2	1.2
102	1.3	1.7	1.6	3.4
110	1.4	0.6	1.3	2.4
101	1.8	2.2	2.2	3.0
130	1.9	0.6	1.6	3.5
123	2.0	2.6	2.5	5.1
112	2.1	3.1	2.7	5.7
111	2.6	3.0	3.0	3.2
114	4.0	1.1	3.3	1.7
118	4.7	4.5	5.1	7.2
124	5.0	3.9	5.1	3.8
108	5.2	2.0	4.5	3.5

Entries are listed from low to high z-scores for T-2 toxin. Satisfactory results are shaded in green, doubtful ones in yellow and unsatisfactory in amber.

#### Identification of sources of variability in the data population:

When plotting kernel density plots for bump-hunting no extraordinary observations were made for the LO and SP material. However in case of the HI material (Figure 4) two clear maxima can be identified indicating two populations. Further investigation led to the conclusion that the factor associated with this effect is derived from the measurements of the rather high levels of HT-2 but not from the T-2 measurements (as mentioned earlier). This is shown in Figure 5. Some of the participants that indicated problems with their calibration and their results (amongst others) cluster around the lower maxima of the kernel density plot.

Grouping results according to various analytical aspects (e.g. extraction solvent used, extraction mode, detection system, or spiking details), did not indicate that these effects were the reason for the observed bimodal distribution. However, when grouping results for the instrumental technique used (GC, ELISA and LC), not only a difference in the dispersion of the results was observed, indicating that GC appears to be the more robust methodology used in this proficiency test (PT) for samples with high contents of HT-2 toxins (Figure 6), but also that LC is the main contributor leading to two populations in this PT.

Figure 4: Kernel density plots for the sum of T-2 and HT-2 toxins of the HI material:

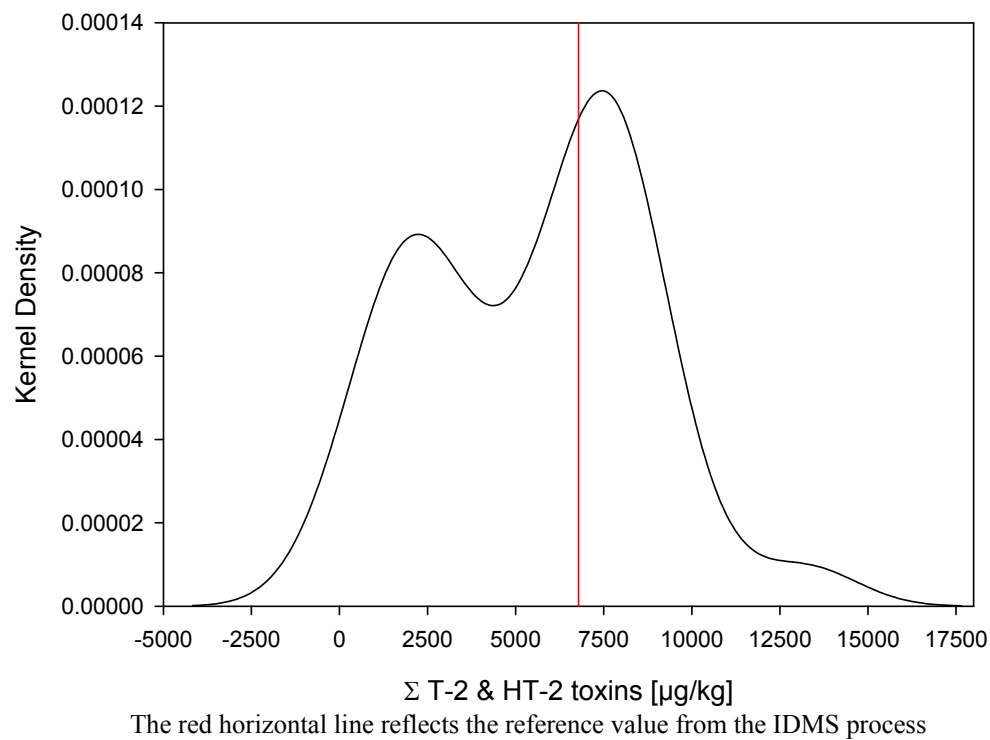


Figure 5: Kernel density plots for T-2 and HT-2 toxins (HI material)

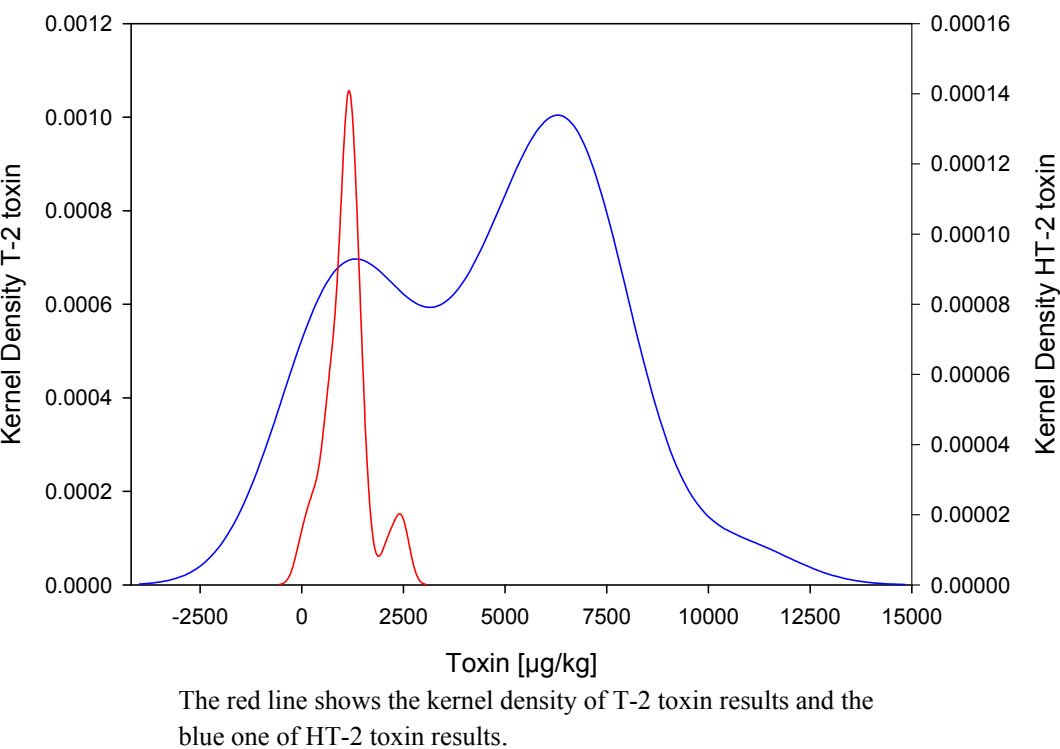
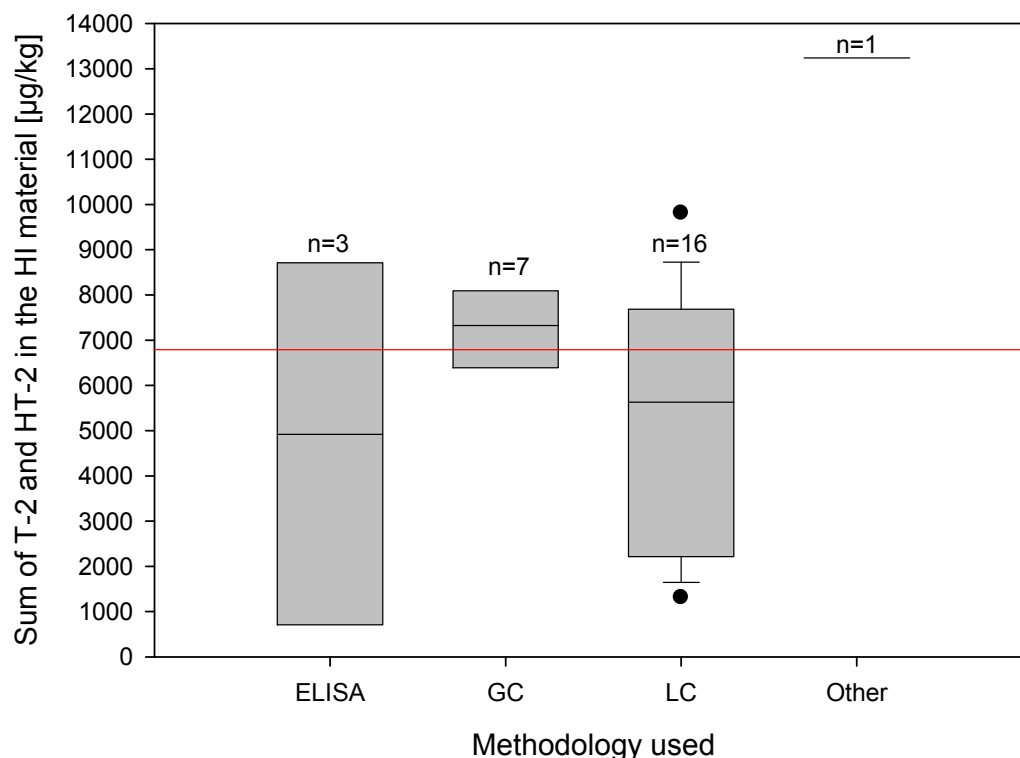


Figure 6: Box and Whisker plots comparing instrumental techniques:



Box plots for the methods used, displaying the median (black horizontal line in the grey box) the upper and lower quartile (25<sup>th</sup> and 75<sup>th</sup> percentile) as box borders and 10<sup>th</sup> and 90<sup>th</sup> percentile as vertical bars. Outliers are displayed as solid points. The red line shows the reference value.

## Conclusion

- z-scoring was used to benchmark the reported results for the spiked material. Four laboratories reported questionable results within a z-score limit of |2| to |3| for T-2 toxin and two laboratories for HT-2 toxin. Five laboratories obtained unsatisfactory z-scores above |3| for T-2 toxin and 11 ones for HT-2 toxin. As a result these laboratories will be asked to repeat their analysis with a new test material. If the result for this test material is still unsatisfactory, the laboratory concerned will be asked to perform a root cause analysis to identify the reason for its poor performance. Based on the outcome further actions will be decided.
- ζ-scoring allows in combination with z-scoring to check for sound estimation of the measurement uncertainty.
- Laboratories, especially those using LC for the determination of T-2 and HT-2 toxins, should be aware of the linear range of their methods and have appropriate procedures in place when highly contaminated samples are analysed.

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# Annex

**Table I:** Individual results as reported by the participants:

Laboratory Code	Low Level Material [µg/kg]					Medium Level Material [µg/kg]					High Level Material [µg/kg]					Recovery Data [%]			
	Code	T-2	HT-2	Σ	U	Code	T-2	HT-2	Σ	U	Code	T-2	HT-2	Σ	U	RecCor	T-2	HT-2	Σ
101	7784	7.6	20.4	28	7	8193	351.6	203.4	554.9	83.2	5785	1258.4	6841.9	8100.2	1244.9	Yes	99	92	-
102	5534	7	11.4	18.4	0.6	4524	322.9	189.8	512.7	15.9	8385	1353.6	6910.2	8263.8	256.2	Yes	87.2	84	85.4
103	2659	7	12	19	-	6642	298.9	173.6	472.5	130	2239	680	1800	2480	530	Yes	91	91	91
104	9274	0	20	20	10.4	6671	313.9	114.7	428.6	194.8	9142	1126.1	2627.6	3753.7	1705.9	Yes	97	99	-
105	5324	6.9	17.7	24.6	2	1155	292.5	147.7	440.2	18.4	4245	1403.1	7307.3	8710.4	190.6	Yes	67	75	71
106	8884	12.1	3.4	15.5		7377	169.7	34.9	204.6	-	5699	422	1161	1583	-	No	63.2	77.7	70.4
107	8421	8	14	22	2.9	2391	320	169	489	68.6	6812	1208	5651	6859	-	No	115	85	100
108	6693	24.1	20.9	44.9	14.6	Spike	524.8	197.2	722	172.9	4195	1459.7	327.7	1787.3	369.7	Yes	73.5	77	-
109	9393	5.5		5.5	2	7977	244.6	350	594.6	50	8335	330.4	380	710.4	50	Yes	126	100	113
110	1563	0	0	0	-	6624	331.8	157	488.8	40.9	5284	1256	5836.4	7092.4	528.1	No	-	-	-
111	5457	3.1	8.9	12.0	4.4	4482	388.7	226.2	614.9	118.7	4451	1024.2	898.7	1922.9	306.8	Yes	53.5	52.5	
112	5323	0	0	0		8356	365.8	230.8	596.4	23.7	1916	1545.4	8263.1	9779.5	1048.4	Yes	80.5	80.4	80.5
113	4646	35.8	-	-	21	9992	236.2	-	-	21	1779	2098.2	-	-	21	No	-	-	-
114	5188	0	0	0	-	4481	464	171	635	267	4268	1115	6931	8046	-	Yes	87	101	95
115	1885	<100	<100			5541	316	172	488	-	8583	1280	6690	7970	-	Yes	-	-	-
116	8551	6.3	10.5	16.8	1.5	1592	241.1	145.5	386.6	45.5	4483	1215.1	5060.2	6275.3	427.5	Yes	100.2	100.5	100.35
117	9536	0	0	0	0	1519	0	0	0	0	6431	0	0	0	0	Yes	0	0	0
118	5632	0	36.38	36.38	1.45	7925	461.85	259.59	721.44	80.85	3451	2227.42	10302.7	12530.12	346.61	No	93	95	95
119	1524	3.1	11	14.1	4.7	9254	181.2	127.1	308.3	67.8	1836	1124.4	5266.8	6391.2	1406	Yes	109.8	104.4	-
120	1852	52.8	58.3	111.1	30	8347	119	362	481	130	2618	2508	4497	7004	1800	Yes	87	76	-
121	6794	5.1	21.5	26.6	-	2635	282.3	144.9	427.2	-	8121	1056.8	3862	4918.8		No	-	-	-
122	2541	10	12	12	3	2218	260	146	406	81.2	8961	1180	5730	6910	829	No	96	94	95
123	7441	7.2	23.6	30.8	1.8	5758	362.5	215.5	578	27.4	1111	1203.6	6885.4	8089	478	Yes	77	91	-
124	7577	3.5	17.4	20.9	15	spike	514.1	255	769.1	184.6	6636	782.5	4197.3	4979.9	1195.2	Yes	100	100	100
125	8627	0.9	5.3	6.2	1.8	3937	321.9	163.5	485.4	137	8445	931.9	2287	3218.9	911	Yes	94.8	86.5	89.1
126	4886	3.9	12.5	16.4	3.2	1542	271	200	471	92	7749	950	1400	2350	458	No	92	104	98
127	7624	20.9	92.7	96.2	50.5	6724	224.6	393.1	576.8	302.8	5873	684	6948.1	6118.6	3212.3	Yes	94.5	57.8	76.2
128	4132	126.2	-	-	-	SPIKE	278.5	-	-	-	1143	244.2	-	-	-	Yes	89.84	-	-
130	4826	0	0	0	0	4287	355.9	155.9	511.8	-	5933	605.3	708	1313.3	-	Yes	-	-	-
131	8818	0	17.4	17.4	2	8483	293.2	169.1	462.3	50.8	2454	1060.3	6001.2	7061.5	776.8	No	95	113.4	104.2

Σ = Sum of T-2 and HT-2 toxins, U =reported (expanded) uncertainty, RecCor = have results been corrected for recovery

**Table II:** Individual results as reported by the participants after taking recovery correction into account:

Laboratory Code	Low Level Material [µg/kg]				High Level Material [µg/kg]				Medium (SPIKED) Level Material [µg/kg]			
	T-2	HT-2	Σ	U	T-2	HT-2	Σ	U	T-2	HT-2	Σ	U
101	7.6	20.4	28.0	7.0	1258.4	6841.9	8100.3	1244.9	351.6	203.4	555.0	83.2
102	7.0	11.4	18.4	0.6	1353.6	6910.2	8263.8	256.2	322.9	189.8	512.7	15.9
103	7.0	12.0	19.0	-	680.0	1800.0	2480.0	530.0	298.9	173.6	472.5	130.0
104	-	20.0	20.0	10.4	1126.1	2627.6	3753.7	1705.9	313.9	114.7	428.6	194.8
105	6.9	17.7	24.6	2.0	1403.1	7307.3	8710.4	190.6	292.5	147.7	440.2	18.4
106	19.1	4.4	23.5	-	667.7	1494.2	2161.9	-	268.5	44.9	313.4	-
107	7.0	16.5	23.4	2.9	1050.4	6648.2	7698.7	-	278.3	198.8	477.1	68.6
108	24.1	20.9	45.0	14.6	1459.7	327.7	1787.4	369.7	524.8	197.2	722.0	172.9
109	5.5	-	5.5	2.0	330.4	380.0	710.4	50.0	244.6	350.0	594.6	50.0
110	-	-	-	-	1256.0	5836.4	7092.4	528.1	331.8	157.0	488.8	40.9
111	3.1	8.9	12.0	4.4	1024.2	898.7	1922.9	306.8	388.7	226.2	614.9	118.7
112	-	-	-	-	1545.4	8263.1	9808.5	1048.4	365.8	230.8	596.6	23.7
113	35.8	-	35.8	21.0	2098.2	-	2098.2	21.0	236.2	-	236.2	21.0
114	0.0	0.0	0.0	-	1115.0	6931.0	8046.0	-	464.0	171.0	635.0	267.0
115	<100	<100	-	-	1280.0	6690.0	7970.0	-	316.0	172.0	488.0	-
116	6.3	10.5	16.8	1.5	1215.1	5060.2	6275.3	427.5	241.1	145.5	386.6	45.5
117	-	-	-	-	-	-	-	-	-	-	-	-
118	-	38.3	38.3	1.5	2395.1	10844.9	13240.0	346.6	496.6	273.3	769.9	80.9
119	3.1	11.0	14.1	4.7	1124.4	5266.8	6391.2	1406.0	181.2	127.1	308.3	67.8
120	52.8	58.3	111.1	30.0	2508.0	4497.0	7005.0	1800.0	119.0	362.0	481.0	130.0
121	5.1	21.5	26.6	-	1056.8	3862.0	4918.8	0.0	282.3	144.9	427.2	-
122	10.4	12.8	23.2	3.0	1229.2	6095.7	7324.9	829.0	270.8	155.3	426.2	81.2
123	7.2	23.6	30.8	1.8	1203.6	6885.4	8089.0	478.0	362.5	215.5	578.0	27.4
124	3.5	17.4	20.9	15.0	782.5	4197.3	4979.8	1195.2	514.1	255.0	769.1	184.6
125	0.9	5.3	6.2	1.8	931.9	2287.0	3218.9	911.0	321.9	163.5	485.4	137.0
126	4.2	12.0	16.3	3.2	1032.6	1346.2	2378.8	458.0	294.6	192.3	486.9	91.8
127	20.9	92.7	113.6	50.5	684.0	6948.1	7632.1	3212.3	224.6	393.1	617.7	302.8
128	126.2	-	126.2	-	244.2	-	244.2	-	278.5	-	278.5	-
130	-	-	-	-	605.3	708.0	1313.3	-	355.9	155.9	511.8	-
131	-	15.3	15.3	2.0	1116.1	5292.1	6408.2	776.8	308.6	149.1	457.7	50.8

Σ = Sum of T-2 and HT-2 toxins, U =reported (expanded) uncertainty.

**Table IIIa:** Evaluation of the Questionnaire:

Lab ID\ Question	Experience	Time of Experience	# of Samples per annum	Instrumental Method	DetectionSystem
101	Y	2 years GC/MS (10 years GC/ECD)	250	GC	MSD
102	Y	Since 2008 by GC-MS. Since February 2009 by LC/MS-MS	170	LC	MS/MS
103	Y	1 year	100	GC	MS
112	N			LC	FLD
113	Y	4 years	40	ELISA	
114	Y	Since about 1995.	50	LC	SRM-transitions (one qualifier and one quantifier per substance). Triple quadrupole mass spectrometer (ThermoFinnigan).
115	Y	>5 years	450	LC	Triple quad MS
116	Y	Participation in the collaborative study on validation of analytical method to determine T2-HT2 Toxins in cereals and baby food by IAC and GC/MS	0	LC	MS-MS
117	Y	3 years	200	ELISA	
118	Y	6 month ago	5	GC	Mass Detection
119	Y	>10 years		LC	Mass Spectrometer
120	Y	3 years	100	LC	MS/MS
121	N			GC	MS ion trap. Full scan : quantification ion 244 for T2 and ion 185 for HT2; qualification ion for T2 : 290,436,185; for HT2 : 275,466
104	Y	>10 years	100	GC	Mass spectrometry (single quadrupole)
122	Y	10 years	150	LC	MS-MS
123	Y	5 years	100	LC	MS/MS
124	Y	5 years	125	LC	MS-MS
125	N			Other	GC- MS-QP 2010, Shimadzu
126	Y	about 3 years	20	GC	MSD
127	Y	2 years	5	LC	MS
128	N			ELISA	
130	N			GC	Mass spectrometry
131	Y	Approx. 3 years	50	GC	GC-MS
105	Y	for 1 year	150	LC	LC-MS/MS
106	N			LC	MS/MS
107	Y	10 years	20	LC	MS/MS
108	Y	6 months	50	LC	FLR
109	Y	2 years	300	LC	Triple quadrupole massspectrometry
110	Y	4 years	100	LC	Mass Spec (Triple Quad)
111	Y	6 months	50	LC	MS/MS

**Table IIIb:** Evaluation of the Questionnaire:

Lab ID\ Question	ExtractionSolvent	ExtractionTime	ExtractionMode	TypeCleanUp	IAC_Mod
101	Acetonitril / Water 84 / 16	2 hours	stirring (magnetic stirrer)	SPE (Mycosep227) and IAC (R-Biopharm Easi Extract T2&HT2)	Y
102	Acetonitrile / Water (84 : 16)	3 minutes	Ultraturrax	Mycosep 225	
103	Acetonitrile-water	1 hour	linear shaking	SPE	
112	Methanol/distilled water (90/10 v/v)	2 hours	Shaking with solvent on Orbital Shaker	Centrifugation, filtration, immunoaffinity columns, change of solvents	Y
113	methanol/water 50/50 v/v	3 min	solid - liquid extraction	nothing	
114	Acetonitrile-water-formic acid 840:160:10.	60 min	Shaking	MultiSep 226	
115	Acetonitril/water/formic acid = 84/16/1 (v/v/v)	2 h	Shaker	No	N
116	Methanol-Water	30 minutes	Shaking	IAC (Rhône-Biopharm)	Y
117	Metanol	3 min			N
118	Acetonitrile and water	5 minute	Ultraturrax	Solid Phase Extraction	N
119	Acetonitrile : water, 84 : 16, v/v AcN : H <sub>2</sub> O : CH <sub>3</sub> COOH 80 : 19 : 1	2 hours	Shaking	Mycosep	
120		3 min	ultr - thurax		N
121	Methanol-water 80-20	30 min	magnetic stirrer	immunoaffinity column R-Biopharm	
104	ACN-water (84:16)	120 min	Shaking	Mycosep #227	
122	acetonitrile:water=84:16	2 hours	horizontal shaker	aluminium oxide-activated charcoal	N
123	ACN/water 84/16 (v/v)	60 min	Shaking with Turbula	Charcoal-celite-aluminium oxide	
124	ACN 79%, HAc 1%, H <sub>2</sub> O 20%	1 hour	overhead agitation	none	
125	MeOH:H <sub>2</sub> O (80:20)	30 min	Shake on a flask shaker	IAC (immunoaffinity columns T2&HT2)	N
126	methanol : water, 80:20 (v/v)	30 minutes	shake	immunoaffinity columns	N
127	84 % acetonitril	30 minutes	shaking with solvent	Filtering followed by clean up by MycoSep Trich-columns	
128	MeOH	10 min.	shake vigorously	filtred	N
130	Methanol/water (80:20, v:v)	30 min	liquid/liquid	IAC	Y
131	80:20 MeOH:UPW 100 ml with 1 g NaCl added to sample	2 min.	High speed (13,000 r.p.m.) using an Ultra Turrax T 25	Immunoaffinity columns from R-Biopharm Rhône Ltd.	N
105	ACN: H <sub>2</sub> O: AcOH = 79:20:1	1 hour	shaking	SPE column (Waters Oasis HLB)	N
106	acetonitrile/water 80/20 % (V/V)	2 minutes	homogenization with Ultra-Turrax	Immuno-affinity column	N
107	Acetonitrile :water; 84 :16	2 hours	Shaking	Mycosep 225	
108	MeOH:H <sub>2</sub> O 90:10	1h 15 min	shaking	IAC	N
109	MeOH : H <sub>2</sub> O (9:1)	30 min	Head over head mixing	IAC	N
110	Acetonitril/water 84/16 v/v	60 minutes	shaking	Mycosep	N
111	methanol/water (9:1)	3 min	ultra turrax	immunoaffinity column	Y



**Table IIIc:** Evaluation of the Questionnaire:

Lab ID\ Question	Fortification Mode	Recovery Details
		Two C13-Standards to the Extract of every sample with automatic correction via the classical internal standard calculation (SIDA)! Use of two C13-Standards (13C24-T-2 Toxin and 13C22-HT-2 Toxin) !
101	13C_2_Extract	Recovery Estimate for the Validation-Process of the Method: Toxin-Standard to the blank material and two C13-Standards to the Extract. CORRECTION of the reported results with this Method Validation-Recoveries Analysis of a reference material (FAPAS)
102	Other	
103	Int_2_sample	
112	BlankSpike	
113	0	
114	BlankSpike	
115	Other	The analysis was performed by a standard addition procedure. No specific recovery was calculated.
116	BlankSpike	
117	BlankSpike	
118	BlankSpike	
119	C13_2_Extract/ BlankSpike	Spike standard added to blank was used to determine the recovery used to correct the results reported in this study although both methods were used during analysis.
120	C13_2_Extract	
121	Other	External standard added to sample : 100 µg/kg added to 25 g
104	Int_2_extract/ BlankSpike	
122	BlankSpike	
123	BlankSpike	
124	BlankSpike	
125	C13_2_Extract	
126	BlankSpike	
127	BlankSpike	Standard to blank sample
128	BlankSpike	
130	Int_2_sample	
131	C13_2_Extract	
105	BlankSpike	
106	Other	Standard to sample
107	Int_2_extract	
108	BlankSpike	
109	Other	Standard was added to the sample at beginning of extraction procedure.
110	C13_2_Extract	Both C13 T-2 and C13 HT-2
111	Int_2_sample	

**Table III d:** Evaluation of the Questionnaire:

Lab ID\ Question	OverNightStep	OvernightDetails	Problems Encountered	Observations	Observation Details
101	Y	All samples; Extraction on Friday – Clean up on Monday – Silylation and GC/MS on Tuesday (because of problems due to the rebuilding of the laboratory)	Y	N	
102-103, 112 -114	N		N	N	
115	Y	In between extraction and measurement.	N	N	
116-117	N		N	N	
118	N		N	Y	3451 sample diluted 1:10 but calculation done accordingly
119	Y	Sample extraction, clean-up & 1st analysis was performed in one day. Crude extracts were stored in a fridge and a further portion cleaned up for analysis to bring within calibration range at a later date.	Y	N	Only the observation noted above, which led us to use external calibration with no effect on the result.
120	N		N	N	
121	Y	For the three samples after extraction and filtration	Y		
104	Y	All samples. Due to instrumental problems observed, the whole sample set had to be re-analysed one week after the original extraction. During this time, all extracts were stored at +4 degrees.	Y	Y	The calibrant provided could not be used to check own calibration. This was because the response for ISTD remained three times lower in the test sample as compared to own standards. This same phenomenon was observed last year for DON test solution (!??).
122-125	N		N	N	
126	N		Y	N	
127	Y	Sample preparation and clean-up one day, LC/MS analysis another day.	Y	N	
128	N		N	N	
130	N		N	N	
131	Y	The sample extracts (80:20 MeOH:UPW solutions) were frozen after filtration until analysis	Y	Y	Sample no. 8818 needed to be filtered 2–3 times with a GFC before the extracts were clear
105	N		N	N	
106	Y	for all samples, after the extraction/purification step and before HPLC	Y	Y	We had to use different MRM transitions than the one described in the literature
107	N		Y	N	
108	N		N	N	
109	N		Y	N	
110	N		N	N	
111	Y	after filtration step	N	N	

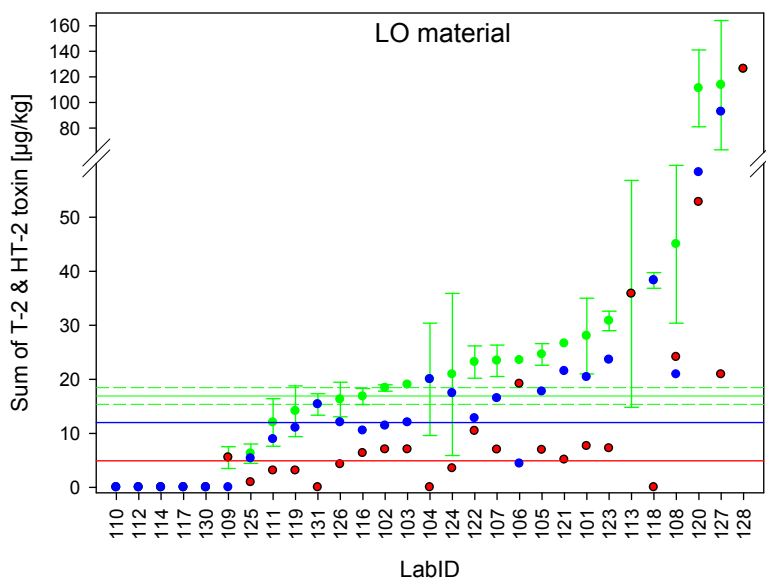
**Table IIIe:** Evaluation of the Questionnaire:

Lab ID\ Question	Integration Mode	Visual Confirmation	Number of Reintegrated Signals	Accreditation?	Were Instructions Adequate?	Any Online Problems?
101	Auto	Y	approx. 20 %	Y	N	N
102	Auto	Y	2	N	N	N
103	Auto	Y	None	Y	N	Y
112	Manual			N	N	N
113	Auto	Y		Y	Y	N
114	Manual			N	Y	N
115	Manual	Y	All chromatograms were corrected	Y	N	Y
116	Auto	Y		N	Y	N
117				N	Y	N
118	Auto	N		N	N	N
119	Auto	Y	n/a	Y	Y	N
120	Auto	N		Y	Y	N
121	Manual	Y		N	Y	N
104	Auto	Y	0	N	N	Y
122	Auto	Y	1	Y	Y	Y
123	Auto	Y	none	Y	N	N
124	Auto	Y	none	N	Y	
125	Auto	Y	Only for peaks of very low concentrations	N	Y	N
126	Manual			Y	Y	N
127	Auto	Y	none	Y	Y	N
128	Manual			N	Y	N
130	Manual			N	N	N
131	Auto	Y	N/A	N	Y	N
105	Auto	N		N	Y	N
106	Auto	Y	2	N	Y	N
107	Auto			Y	Y	N
108	Auto	Y	We needed to re- integrate all HT2 chromatograms	N	Y	N
109	Auto	Y	One.	N	Y	N
110	Auto	Y	none	Y	Y	N
111	Manual			Y	Y	N

**Table IIIf:** Evaluation of the Questionnaire:

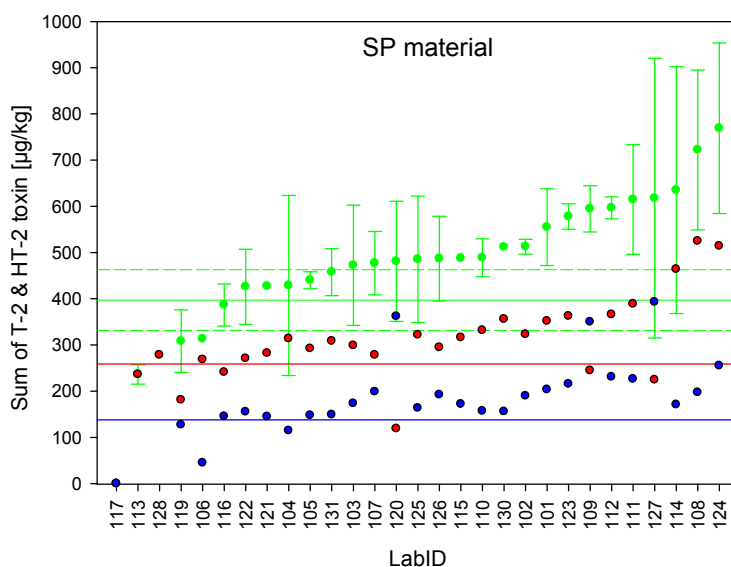
Lab ID\ Question	Opinion on Online Form	Other Comments?
101	o.k.	
102	OK	The e-mail from JRC announcing that the provided calibrant had a known concentration and should be used, did not reach to us in due time. We knew about it when the analysis were finished. The results had to be corrected, consequently.
103	There are some troubles e.g. zero after the decimal coma is not accepted, results under limits are not accepted (e.g. <LOD or n.d.).	
112	The questions and more adequate answers could be.	
113	it is OK	
114	OK	
115	The reporting format is to strict, see also remarks at 19.	
116		
117	The reporting format by electronic forms is the good one.	
118	excellent	
119		
120	OK	
121	It is OK	
104	Good, but the use of special characters should be allowed. E.g. it was not possible to report concentrations below LOQ (now stated as the value of the LOQ).	
122	Sometimes there are submitting problems	
123	OK	
124	easy, no problem	
125	OK	
126	normal	
127	OK	
128	OK	
130	Very convenient	
131	The format is excellent—we have no problem with it	
105	user friendly	
106	It's OK!	
107	ok	
108	They are easy and fast	
109	Very easy!	
110	good	
111		

Figure I: Reported results for the LO material with respect to the single results for T-2 and HT-2 toxins and the values from the certification process:



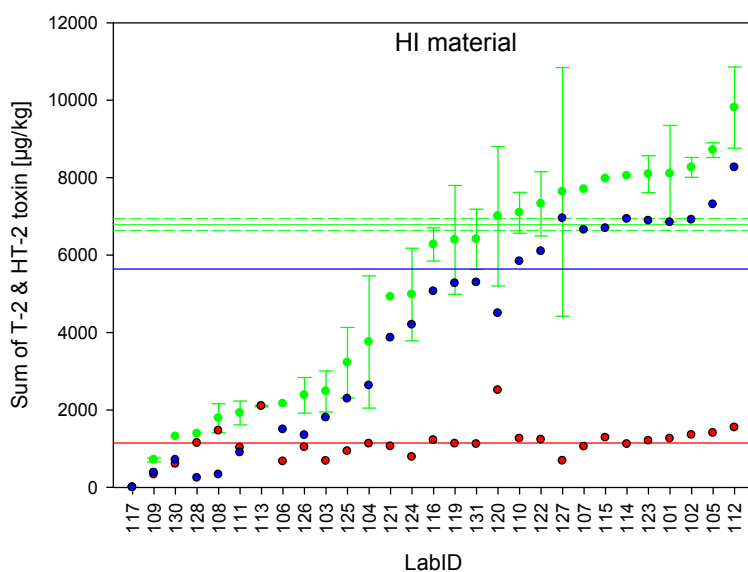
T-2 results are plotted as red dots (●), HT-2 results as blue dots (●) and the sum of T-2 and HT-2 as green dots (●) with the bars indicating the reported uncertainty. The red line reflects the reference value from the IDMS process for T-2, the blue line the corresponding one for HT-2 and the green solid line the corresponding one for the sum of both. The dashed green lines show the uncertainty calculated for the reference value.

Figure II: Reported results for the SP material with respect to the single results for T-2 and HT-2 toxins and the values from the certification process



T-2 results are plotted as red dots (●), HT-2 results as blue dots (●) and the sum of T-2 and HT-2 as green dots (●) with the bars indicating the reported uncertainty. The red line reflects the reference value from the IDMS process for T-2, the blue line the corresponding one for HT-2 and the green solid line the corresponding one for the sum of both. The dashed green lines show the uncertainty calculated for the reference value.

Figure III: Reported results for the HI material with respect to the single results for T-2 and HT-2 toxins and the values from the certification process



T T-2 results are plotted as red dots (●), HT-2 results as blue dots (●) and the sum of T-2 and HT-2 as green dots (●) with the bars indicating the reported uncertainty. The red line reflects the reference value from the IDMS process for T-2, the blue line the corresponding one for HT-2 and the green solid line the corresponding one for the sum of both. The dashed green lines show the uncertainty calculated for the reference value.

## CRL for Mycotoxins 2009 PT:

### Certification of T-2/ HT-2 toxin levels in cereal mixes

#### 1. DETERMINATION OF ASSIGNED VALUES

##### Materials:

The low level (LO) was a naturally contaminated material used previously (2006) during a collaborative study. The medium level (SP) was a blank cereal mix spiked with a defined amount of T-2 & HT-2 toxin. The high level (HI) was naturally contaminated wheat.

$^{13}\text{C}_{24}$  T-2 toxin,  $^{13}\text{C}_{22}$  HT-2 toxin, T-2 toxin, and HT-2 toxin in acetonitrile (Biopure, Tulln, AU) were used to prepare the different sample and calibration blends.

##### Preparation of blends:

For each of the four materials three units (containers) were selected to be used for the determination of the assigned values. After thorough mixing of each test unit two 1 g test portions were removed resulting in 6 test portions per material (see Table 1).

**Table 1:** Weights (g) of the different test portions used for the sample blends; per test unit (3) two test portions (A,B) were removed

Material	Test unit					
	1		2		3	
	A	B	A	B	A	B
LO	1.005	1.001	1.004	1.002	1.005	1.003
SP	1.005	1.008	1.008	1.004	1.000	1.006
HI	1.002	1.005	1.004	1.006	1.008	1.006

From those test portions sample blends (SB) were prepared by adding the volumes of isotopically labelled analytes indicated in Table 2:

**Table 2:** Volumes of isotopically labelled analytes added to the different sample and calibration blends

<b>Material</b>	<b><math>^{13}\text{C}_{24}</math> T-2 toxin [<math>\mu\text{L}</math> (<math>\mu\text{g/mL}</math>)]</b>	<b><math>^{13}\text{C}_{22}</math> HT-2 toxin [<math>\mu\text{L}</math> (<math>\mu\text{g/mL}</math>)]</b>
LO	80 (0.30)	100 (0.50)
SP	100 (3.0)	100 (1.7)
HI	120 (3.0)	80 (25)

The blank cereal mix which was used for the spiked PT material was also used for the calibration blends (CB). For each test material two independent CBs were prepared by adding the same volumes of isotopically labelled analytes as for the SBs (Table 2) plus the volumes of T-2 and HT-2 toxin reference material indicated in Table 3:

**Table 3:** Volumes of reference material added to the different calibration blends

<b>Material</b>	<b>T-2 toxin [<math>\mu\text{L}</math> (<math>\mu\text{g/mL}</math>)]</b>	<b>HT-2 toxin [<math>\mu\text{L}</math> (<math>\mu\text{g/mL}</math>)]</b>
LO	18 (0.30)	26 (0.50)
SP	100 (3.1)	100 (1.7)
HI	405 (3.1)	226 (25)

After addition of the reference materials and isotopically labelled analytes the blends were left at room temperature for 2h to allow the spikes to penetrate the material and the solvent to evaporate. Then 4 mL of methanol/ water (80/20, v/v) were added and the blends extracted by vertical shaking for another 2h. After the extraction the blends were centrifuged for 10 min at RCF 3200 and either 0.5 (SP, HI) or 1 (all LO) mL of the clear supernatant were transferred into deactivated glass vials. The aliquots were evaporated to dryness at 60 °C under a gentle stream of nitrogen. The dry residues were reconstituted with 100  $\mu\text{L}$  methanol and vortexed. Then 900  $\mu\text{L}$  water were added and the vial vortexed again. The resulting injection solution was then transferred into ALS vials.

### Measurements:

Measurements were performed on a TSQ Quantum Ultra (Thermo Scientific) connected to a binary high-pressure solvent delivery system (LC-20AD, Shimadzu) and an Accela auto liquid sampler (Thermo Scientific). Separation was afforded by an Ascentis C18 express column (75 x 2.1 mm, 2.7  $\mu\text{m}$ ) with a mobile phase of 0.1 % formic acid in water (A) and 0.1% formic acid in methanol (B). Gradient settings were such that apparent retention factors of 10 (HT2 toxin) and 14 (T-2 toxin) were obtained.



Electro spray ion source settings were as follows: spray voltage 2800/2400 kV for HT-2 and T-2 toxin, respectively, vaporizer temperature 350 °C, capillary temperature 320 °C, sheath gas 30, ion sweep gas 10.0, aux gas 10 (gas pressures in arbitrary units).

In selected reaction monitoring mode the sodium-adducts of the parent compounds were selected for the following transitions: 447.2→345.1 for HT-2 toxin, 469.2→362.0 for <sup>13</sup>C<sub>22</sub>-HT-2 toxin, 489.2→245.0 for T-2 toxin, and 513.2→260.1 for <sup>13</sup>C<sub>24</sub>-T-2 toxin. The dwell times were chosen such that about 20 scans across a peak were registered.

Batches of runs were structured such that a CB run was followed by a SB run. The two CB preparations were constantly alternated. This was repeated so that each SB was injected five times with its corresponding CB.

### Calculation of the assigned values and their uncertainties

The following model equation was used:

$$w_{s,i} = c_{c,i} \times \frac{V_{c,i} A_{ISTD,CB}}{V_{ISTD,CB} A_{Analyte,CB}} \times \frac{V_{ISTD,SB} A_{Analyte,SB}}{m_{smp,i} A_{ISTD,SB}} \quad (1)$$

with

$w_{s,i}$	=	mass fraction of analyte in test portion
$c_{c,i}$	=	concentration of analyte in the reference solution
$V_{c,i}$	=	volume of the reference solution
$V_{ISTD,CB}$	=	volume of the ISTD solution added to CB
$V_{ISTD,SB}$	=	volume of the ISTD solution added to SB
$m_{smp,i}$	=	mass of test portion
$A_{Analytel,SB}$	=	Peak area of analyte in SB
$A_{ISTD,SB}$	=	Peak area of labelled analyte in SB
$A_{Analytel,CB}$	=	Peak area of analyte in CB
$A_{ISTD,CB}$	=	Peak area of labelled analyte in CB

For each corresponding pair of a CB and a SB run  $w_{s,i}$  was calculated as above. The assigned value was calculated as the average of all  $w_{s,i}$  per material:

$$x_a = \bar{w}_{s,i} \times F_{BS} \quad (2)$$

The uncertainty of  $x_a$  is then given by:

$$u(x_a) = x_a \times \sqrt{\frac{\sum u^2(w_{s,i})}{nx_a^2} + \frac{u^2(F_{BS})}{x_a^2}} \quad (3)$$

In Equ. 2 the term  $F_{BS}$  has a value of 1 and accounts for the uncertainties due to the between-samples variability. This variability includes amongst others inhomogeneity, instrument precision, precision of the volume measurements, and precision of the weighings. It is calculated as the standard error of the mean of the 6 SBs per material. Table 4 lists the results:

**Table 4:** The assigned values and the associated uncertainties

Material	Analyte	Assigned value ( $x_a$ ) [mg/kg]	Expanded Uncertainty ( $U(x_a)$ ) [mg/kg, (%)]	Coverage factor (k)	Principal contributors [Name, (%)]
LO	T-2	4.92	0.57 (12)	2	$u_{BS}$ (77%), $c_{c,i}$ (22%)
	HT-2	12.0	1.42 (12)	2	$u_{BS}$ (55%), $c_{c,i}$ (45%)
	SUM	16.9	1.53 (9)	2	$u_{BS,HT2}$ (47%), $c_{c,HT2}$ (39%), $u_{BS,T2}$ (11%)
SP	T-2	258.9	59.2 (23)	2	$u_{BS}$ (99%)
	HT-2	138.2	28.2 (20)	2	$u_{BS}$ (97%)
	SUM	397.1	65.6 (17)	2	$u_{BS,T-2}$ (81%), $u_{BS,HT-2}$ (18%)
HI	T-2	1144	21 (2)	2	$c_{c,i}$ (54%), $u_{BS}$ (28%), $V_{c,i}$ (11%)
	HT-2	5642	151 (3)	2	$c_{c,i}$ (85%), $u_{BS}$ (7%), $V_{c,i}$ (7%)
	SUM	6787	153 (2)	2	$c_{c,HT2}$ (84%), $u_{BS,HT2S}$ (7%), $V_{c,HT2}$ (6%)

The stated expanded uncertainties were calculated under repeatability conditions, except for LO which was measured on two different days. There is an unknown bias to the assigned value of LO because the SB/CB ratio was around 0.3 on day 1 and 0.8 on day 2. This component is not included in the estimate of the expanded uncertainty. The main driver for the high uncertainties of the SP material is the significant inhomogeneity between the test units.

European Commission

**EUR 24315 EN – Joint Research Centre – Institute for Reference Materials and Measurements**

Title: Report of the 2009 Proficiency Test of the Community Reference Laboratory for Mycotoxins, for the Network of National Reference Laboratories, Regarding the Determination of T-2 and HT-2 Toxins in Cereal Products

Authors: J. Stroka, A. Breidbach, K. Bouten, K. Kroeger, M. Ambrosio, D. Lerda

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**Abstract**

A proficiency test was conducted by the Community Reference Laboratory for Mycotoxins with 29 European National Reference Laboratories (NRLs) for Mycotoxins and 1 Laboratory from a candidate country. The materials shipped were a solution of known T-2 and HT-toxin content in acetonitrile and three cereal test materials with unknown levels of T-2 and HT-2 toxin. Laboratories determined the content of T-2 and HT-toxins by either enzyme linked immuno sorbent assay (ELISA), gas chromatography (GC-MS) or high-performance liquid-chromatography (HPLC) followed by fluorescence or mass selective detection (MS). From each Member State (MS) the NRL reported results, with two MS reporting results from a feed and a food NRL.

Horwitz equation was applied as a basis for setting the target standard deviation for proficiency (19.6% for T-2 toxin and 21.5% for HT-2 toxin in the spiked test material). 21 laboratories out of the 30 participating reported satisfactory z-scores for T-2 toxins and 15 laboratories for HT-2 toxins (after recovery correction). Two laboratories did not send in results for HT-2 toxin, but only for T-2 toxin. Four laboratories reported questionable results within a |z-score| between 2 and 3 T-2 toxin and 2 laboratories for HT-2 toxin. The remaining laboratories reported |z-score| above 3, which are unsatisfactory. Taking the  $\zeta$ -score as benchmark for the combined parameter (T-2 & HT-2 toxin) the number of satisfactory results reduced to 14. No z-scores were calculated for the low contaminated and the high contaminated material.



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